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ISOLATION OF A SYNAPTIC MEMBRANE FRACTION ENRICHED IN CHOLINERGIC RECEPTORS BY CONTROLLED PHOSPHOLIPASE A_2 HYDROLYSIS OF SYNAPTIC MEMBRANES

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SUMMARY

A procedure is described for the isolation of synaptic membrane fragments that retain such functionally important proteins as acetylcholine receptors, acetylcholinesterase, 3′,5′-cyclic nucleotide phosphodiesterase, and $(Na^+ + K^+)$-ATPase. The method is based on the observation, made in brain slices, that junctional membranes are more resistant to phospholipase A_2 attack than mitochondrial or plasma membranes. Hydrolysis by phospholipase A_2 was controlled by addition of fatty acid-free bovine serum albumin. The membrane fraction obtained represents approximately a 15-fold enrichment of the postsynaptic marker proteins muscarinic and nicotinic acetylcholine receptor and 3′,5′-cyclic nucleotide phosphodiesterase over an ordinary synaptic plasma membrane preparation, and is devoid of mitochondrial and microsomal contaminations. The membranes appear on the electron micrographs as rigid fragments (average length 2500–4000Å), which do not form vesicles.

INTRODUCTION

Biochemical studies of the synaptic region require reasonably pure subcellular fractions of this highly specialized contact area between neurons. Consequently, techniques have been devised for the isolation of synaptosomes [1–3], a subcellular fraction which permits biochemical and structural studies of the presynaptic mechanisms, and for the isolation of synaptic junctional complexes [4–7] which have been studied mostly from a structural point of view. No functional muscarinic postsynaptic membrane preparations are available. Current theories about mechanisms of action at muscarinic postsynaptic sites are therefore mainly deduced from pharmacological and neurophysiological work. The most probable sequence of reactions at the muscarinic synaptic membrane may be summarized as follows: binding of acetylcholine to a receptor, synthesis of cyclic nucleotides [8], activation of cyclic nucleotide-dependent protein kinases [9] that phosphorylate intrinsic membrane proteins [10], change of membrane permeability and removal of the transmitter from the receptor. A closer study of the steps leading to membrane permeability changes would require prep-

arations of postsynaptic membranes that are functionally intact (i.e. retain all the above-mentioned enzymes in an active state). A main problem when attempting the preparation of such membranes is the firm attachment of the pre- to the postsynaptic membranes. Also the postsynaptic structure in itself is complex, involving a specialized plasma membrane and a protein specialisation, postsynaptic density [11], associated with it. Recently, isolation of postsynaptic densities was achieved by solubilization of most of the plasma membranes by *N*-dodecyl sarcosinate. The aim of the present study was to purify the membrane part of the postsynaptic structure, which we assume is the locus of the neurotransmitter receptors and of other proteins involved in postsynaptic events.

A method based on a controlled hydrolysis of the synaptosomal membrane fraction by purified phospholipase A_2 is described. Previous ultrastructural studies [12, 13] showed that incubation of brain slices with phospholipase A_2 leads to ruptures of the plasma membranes, whereas the synaptic junctions appear to be intact after the treatment. However, on prolonged incubation the presynaptic membranes are also attacked. It was also known that addition of fatty acid-free bovine serum albumin abolishes the detergent effects of nascent lysophosphatidylcholine [14]. Based on these observations, a method is now devised which gives a membrane preparation enriched in acetylcholine receptors and 3′,5′-cyclic nucleotide phosphodiesterase.

MATERIALS AND METHODS

The enzyme reagents and substrates used were obtained from Sigma Chemicals. Phospholipase A_2 was purified from the venom of *Naja naja siamensis* by Dr. E. Karlsson, Uppsala. The preparation was free from proteolytic activity.

Isolation of postsynaptic membranes

Adult male rats (250 g body weight) were decapitated in a cold room (4 °C). All the subsequent steps of the isolation procedure were carried out at 4 °C. The forebrain was removed and homogenized at 100 rev./min in a Potter-Elvehjem homogenizator and the homogenate was submitted to the centrifugation steps summarized in Fig. 1. The 10 % (w/v) homogenate in sucrose was centrifuged at $1000 \times g$ for 25 min. The pellet was resuspended in 10 % sucrose and centrifuged again and the supernatants were combined to give fraction S. This fraction was pelleted at $10\,000 \times g$ for 35 min. The resulting crude mitochondrial pellet was resuspended in 10 % sucrose and pelleted again. Preparation of synaptosomal membrane from the crude mitochondrial pellet was carried out according to the flotation-sedimentation method of Jones and Matus [3]. To remove sucrose the synaptosomal membranes obtained (fraction II) were pelleted ($100\,000 \times g$, 60 min) and resuspended in phosphate buffer (10 mM, pH 7.5) to give a protein concentration of about 100 mg/ml. Aliquots were taken from this fraction and incubated with phospholipase A_2 for 30 min at 30 °C.

The action of phospholipase A_2 was stopped by the addition of 100 mg EDTA to the reaction mixture, which then was layered on a discontinuous sucrose gradient (0.8, 1.0 and 1.2 M) containing EDTA (0.1 mg/ml) and centrifuged at $50\,000 \times g$ for 12 h. Two protein bands were obtained at the 0.8-1.0 M and 1.0-1.2 M interfaces,

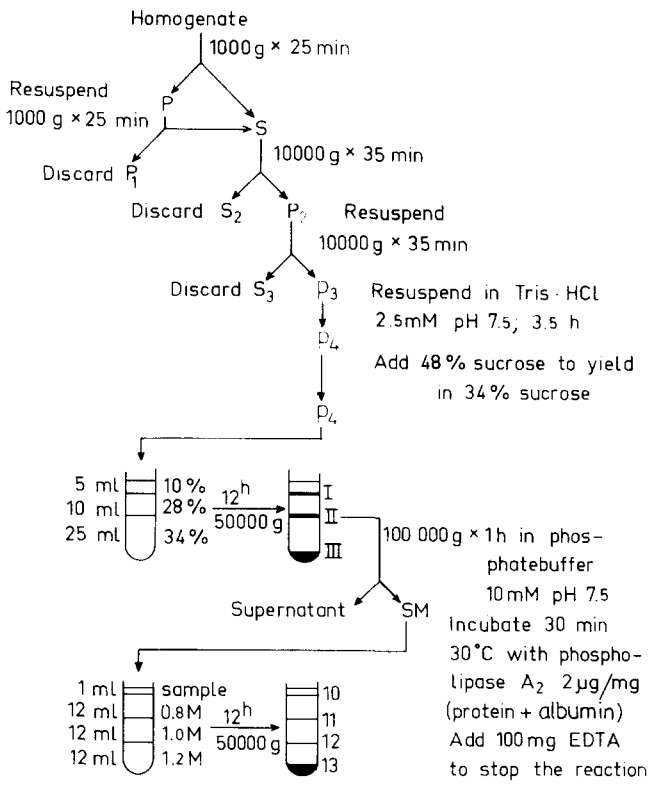

Fig. 1. Preparation scheme for the isolation of synaptic membranes enriched in postsynaptically located proteins. The centrifugation steps indicated were carried out in a Beckman ultracentrifuge. Preparation of synaptic membranes (Fraction II) from lysed crude mitochondrial pellet and the subsequent centrifugations were carried out using swing-out rotors SW 27 and SW 40, whereas the other steps were carried out with a 60 Ti fixed angle rotor.

respectively, and a pellet was formed. These fractions were denoted as 11, 12 and 13. The effect of changing the phospholipase A_2 to protein ratio on the disruption of the membranes was studied by the method described above. Efforts were made to separate the membrane fragments resulting from the phospholipase A_2 treatment from contaminating liposomes and vesicles by subjecting them to gel filtration on a 30 cm long, 2 cm external diameter Sepharose 2B column. The gel filtration was carried out in phosphate buffer (10 mM, pH 7.5). The density of the postsynaptic membrane fragments was determined by gradient centrifugation on a continuous sucrose gradient (1.1–1.3 M) in an SW-56 Beckman rotor (50 000 × *g*, 3 h).

Enzyme and receptor assays

The purity of the fractions was assessed by marker enzyme studies and by electron microscopic characterization. As markers for the cholinergic postsynaptic membranes, only the muscarinic and nicotinic acetylcholine receptors were used as the 3′,5′-cyclic nucleotide phosphodiesterase appears both in cholinergic and in adrenergic postsynaptic membranes [15] and acetylcholinesterase is present in soluble and membrane-bound forms in the brain and the membrane-bound enzyme is abundant on

both sides of the synaptic cleft [16]. The rotenone-sensitive and insensitive cytochrome *c* reductases were used as mitochondrial [17] and microsomal [17] markers, respectively. Lactate dehydrogenase was regarded as a cytosolic marker [17] and succinate dehydrogenase as an additional marker of mitochondrial membranes [17].

The $(Na^+ + K^+)$-activated ATPase is a general plasma membrane marker; however, this enzyme also has a specific role in permeability changes of the postsynaptic membrane, although it has not been proven that the enzyme concentration is higher in those regions than in the plasma membrane. To our knowledge there is no specific marker for presynaptic membrane.

ATPase was measured by the spectrophotometric method of Asami et al. [18]. Acetylcholinesterase was assayed according to Ellman et al. [19]. The rotenone-sensitive and insensitive cytochrome *c* reductases were determined according to Duncan et al. [20]. 3′,5′-cyclic nucleotide phosphodiesterase was measured by the method of Breckenridge and Johnston [21]. The amount of muscarinic acetylcholine receptor was determined from [^{3}H]atropine binding experiments [22], whereas the nicotinic acetylcholine receptor was assayed by means of binding studies with [^{3}H] acetyl-α-neurotoxin from *N. naja siamensis* [23]. When the binding studies were carried out in the presence of bovine serum albumin, possible binding to this protein was taken into consideration. However, α-neurotoxin is highly specific for the nicotinic acetylcholine receptor and specificity of toxin binding was routinely checked by competition between the toxin and α-tubocurarine or acetylcholine. [^{3}H]Atropine was used in 10^{-8} M concentration in a high ionic strength medium, which prevents nonspecific binding to proteins other than the muscarinic acetylcholine receptor. The specificity of the binding to the muscarinic receptor was checked routinely by measuing depression of the atropine binding by other cholinergic ligands (muscarine 10^{-6} M or acetylcholine 10^{-5} M in the presence of physostigmine 10^{-4} M). Protein was determined according to Kalckar et al. [25] and inorganic phosphate according to Ames [26].

Electron microscopy

The samples were pelleted and prefixed in 4 % OsO_4 for 1 h before fixation in 12.5 % glutaraldehyde. Before embedding, the sections were stained with uranyl acetate (2 %). After sectioning, the samples were stained with lead citrate and examned in a Philips 201 Mc electron microscope.

RESULTS

The synaptic membrane fraction obtained by the method of Jones and Matus [13] contains many well preserved synaptic junctions (Fig. 2). Using this method our experiments showed that the speed of the first homogenization step is of great importance for the final isolation of membranes enriched in postsynaptic markers. In four preparations the number of preserved synaptic junctions in fraction II was found to be inversely proportional to the velocity of homogenization. Fig. 2 shows a fraction obtained from material homogenized at 100 rev./min. Contamination by myelin or mitochondria is very low as seen on the electron micrograph.

Table I shows that mitochondrial marker activities are still measurable, though much decreased, in the synaptic membrane preparation. Acetylcholinesterase, the

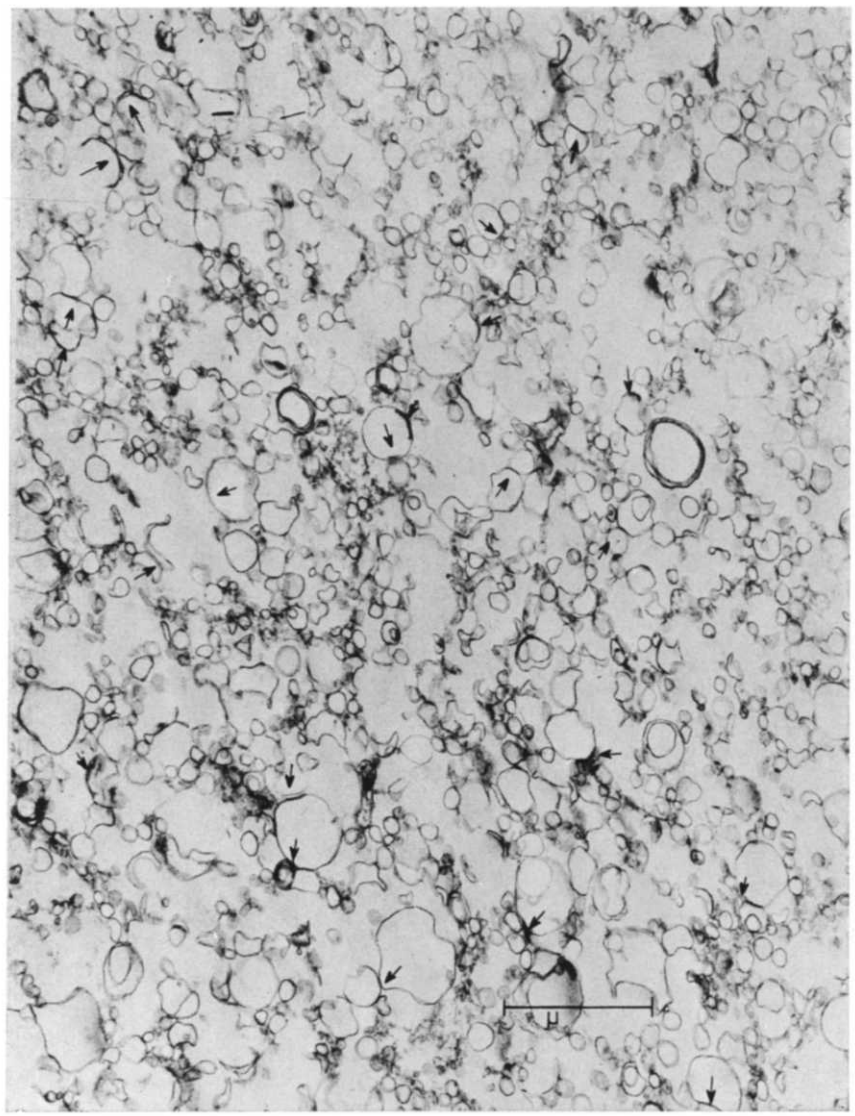

Fig. 2. Electronmicrograph of the synaptic membrane fraction (II). The arrows indicate synaptic densities.

nicotinic acetylcholine receptor, 3′,5′-cyclic nucleotide phosphodiesterase and ($Na^+ + K^+$)-activated ATPase were enriched in fraction II, whereas the specific binding capacity of the muscarinic acetylcholine receptor is slightly decreased, as compared to the homogenate. The decrease in the specific activity of the muscarinic receptor is due to the fact that a great part of this protein is attached to membranes which sediment only at $100\,000 \times g$ for 1 h [22]. As preparation of synaptic membrane fraction II uses the $10\,000 \times g$ pellet, a loss in muscarinic receptor is observed.

To determine the optimal ratio of phospholipase A_2 to total protein, several experiments were carried out. Fig. 3 shows that a ratio of 2 μg phospholipase A_2/mg

TABLE I

SPECIFIC ACTIVITIES OF THE MARKER PROTEINS IN THE FRACTIONS OBTAINED WHEN PREPARING SYNAPTIC MEMBRANES (FRACTION II)

Marker	Fraction		
	Homogenate	Crude mitochondrial pellet	II
Protein (mg/ml)	82.2	112.0	96.0
Muscarinic acetylcholine receptor (pmol/mg)*	1.11 ±0.18	0.97 ±0.08	0.90 ±0.12
Nicotinic acetylcholine** + receptor (pmol/mg)	0.015±0.002	0.065±0.002	0.099±0.003
3′,5′-cyclic nucleotide phosphodiesterase (μmol/mg per min)	115.0 ±5.8	142.0 ±6	157.1 ±5
Acetylcholinesterase (mmol/mg per min)	0.017±0.001	0.015±0.001	0.033±0.002
$(Na^+ + K^+)$-ATPase (μmol/mg per min)	0.62 ±0.04	1.24 ±0.03	0.98 ±0.03
Succinate dehydrogenase (μmol/mg per min)	3.3 ±0.12	4.9 ±0.2	0.8 ±0.07
NADH-cytochrome *c* reductase, rotenone sensitive (μmol/mg per min)	0.022±0.005	0.051±0.005	0.006±0.006
NADH-cytochrome *c* reductase rotenone insensitive (μmol/mg per min)***	0.006±0.004	0.004±0.003	0.001±0.003
Lactate dehydrogenase (μmol/mg per min)	0.039±0.006	0.014±0.006	0.031±0.009

* Calculated from specific atropine binding [22].

** Calculated from toxin binding, assuming two toxin-binding sites per receptor molecule.

*** These results are from two experiments, whereas the other data are derived from 3–4 experiments.

total protein per ml is optimal, as most of the marker proteins remained attached to the denser (> 1.2 M sucrose) membrane fraction after this treatment. Use of this ratio delipidates the membranes only slightly; about 20 % less phosphorus was found on a protein basis than in the synaptosomal membrane fraction. When more phospholipase A_2 is used, some of the membrane fragments become thinner and shift to lower densities, whereas another part, as judged from phosphorus determinations, is delipidated and becomes denser than 1.4 M sucrose. Control of the phospholipase A_2 to protein ratio is therefore of great importance.

Bovine serum albumin was succesfully used to improve the phospholipase A_2 to protein ratio, thereby controlling the hydrolysis of the membranes. The effect of albumin is a dual one: it controls the detergent effects of the formed lysophosphatidylcholine by adsorbing the latter and preventing formation of micelles [4]. Beside this effect, albumin is also assumed to be able to adsorb the phospholipase A_2 [4], which produces lysophosphatidylcholine.

The optimal ratio of 2 μg phospholipase A_2/mg total protein per ml was used for incubation of the synaptic membrane fraction II. After stopping the action of

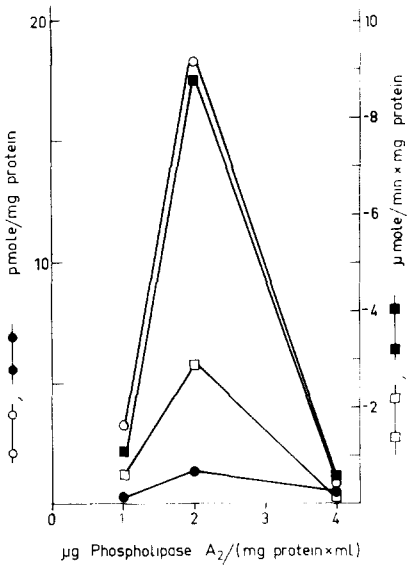

Fig. 3. The effect of phospholipase A_2/total protein ratio on the solubilization of some marker proteins. After incubation with different phospholipase A_2/protein ratios, the mixture was layered onto a discontinuous gradient. A lighter (1.0-1.2 M sucrose interface) and a denser (1.2-1.4 M sucrose interface) membrane fraction was collected. The figure shows the specific activities of the marker proteins which remain associated with the denser membrane fraction. Muscarinic acetylcholine receptor (○), nicotinic acetylcholine receptor (●), $(Na^+ + K^+)$-ATPase (■) and 3′,5′-cyclic nucleotide phosphodiesterase (□).

phospholipase A_2 by addition of EDTA, the synaptic membranes were subfractionated on a discontinuous sucrose density gradient. Three subfractions were recovered from the gradient, one from the 0.8-1.0 M sucrose interface (fraction 11), the second from the 1.0-1.2 M (fraction 12) sucrose interface, and the third from the bottom of the tube (fraction 13). The electron micrographs (Figs. 4 and 5) of fractions 12 and 13 revealed the presence of membrane bars with an average length of 2500–4000 Å, some of them densely coated. The membranes of fraction 13 are thicker than those of fraction 12. Some membrane bars seem to dissociate at their ends, possibly indicating separation of a junctional complex. None of the fractions contains more than a few vesicles or micelles. In contrast, fraction 11 contains vesicles, lipid micelles and a few membrane fragments.

A quantitative survey of a section of Fig. 5 corresponding to a 20 μm^2 area shows that more than 80 % of the identified structures are membrane fragments (cf. fraction 13). Some non-identified globular structures that do not stain at the boundary are probably micelles of lysophosphatidylcholine and other phospholipids.

The density of subfraction 13 was determined on a continuous sucrose gradient and was found to be equivalent to the density of about 1.24–1.27 M sucrose .

Sepharose 2B-gelfiltration of fraction 12 or of fraction 13 resulted in two protein peaks. The first one consisted almost exclusively of membrane fragments, whereas the second contained vesicles and micelles. The recovery of the synaptic membrane fragments was low (10–15 %), probably because of steric hindrance during the gel filtration.

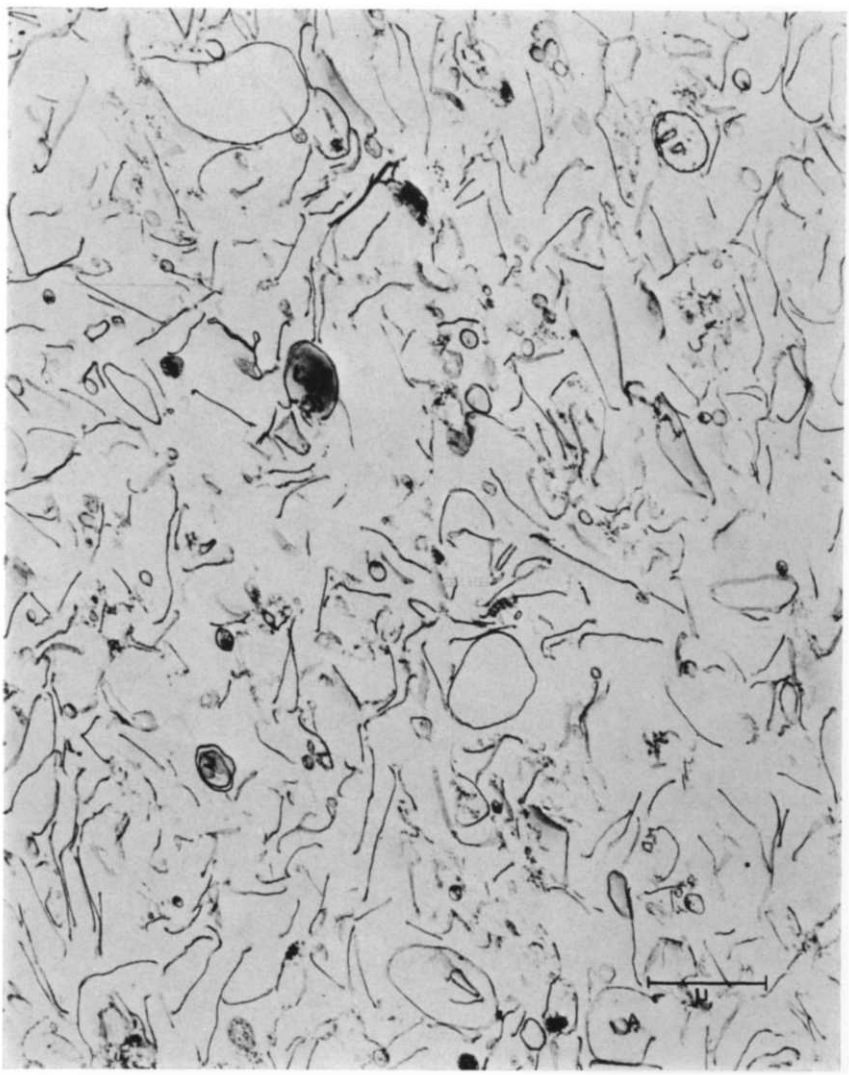

Fig. 4. Electronmicrograph of the phospholipase A_2-treated synaptic membranes. This fraction (12) was harvested from the 1.0-1.2 M sucrose interface when the hydrolyzed membranes were centrifuged.

The specific activities of the marker proteins in fractions 11, 12 and 13 are shown in Table II. Both types of acetylcholine receptor are enriched in fraction 13 as compared to the synaptic membrane fraction II. The same can be said about 3′,5′-cyclic nucleotide phosphodiesterase, acetylcholinesterase and $(Na^+ + K^+)$-ATPase. Protein content, microsomal and mitochondrial contaminations have decreased and the cytoplasmic contamination is unchanged when fractions 13 and II are compared. It is also seen in Fig. 6 that much of the muscarinic acetylcholine receptors, as characterized by atropine binding, and of the 3′,5′-cyclic nucleotide phosphodiesterase is solubilized and appears in the lighter fraction 11. Recovery of the synaptic membrane

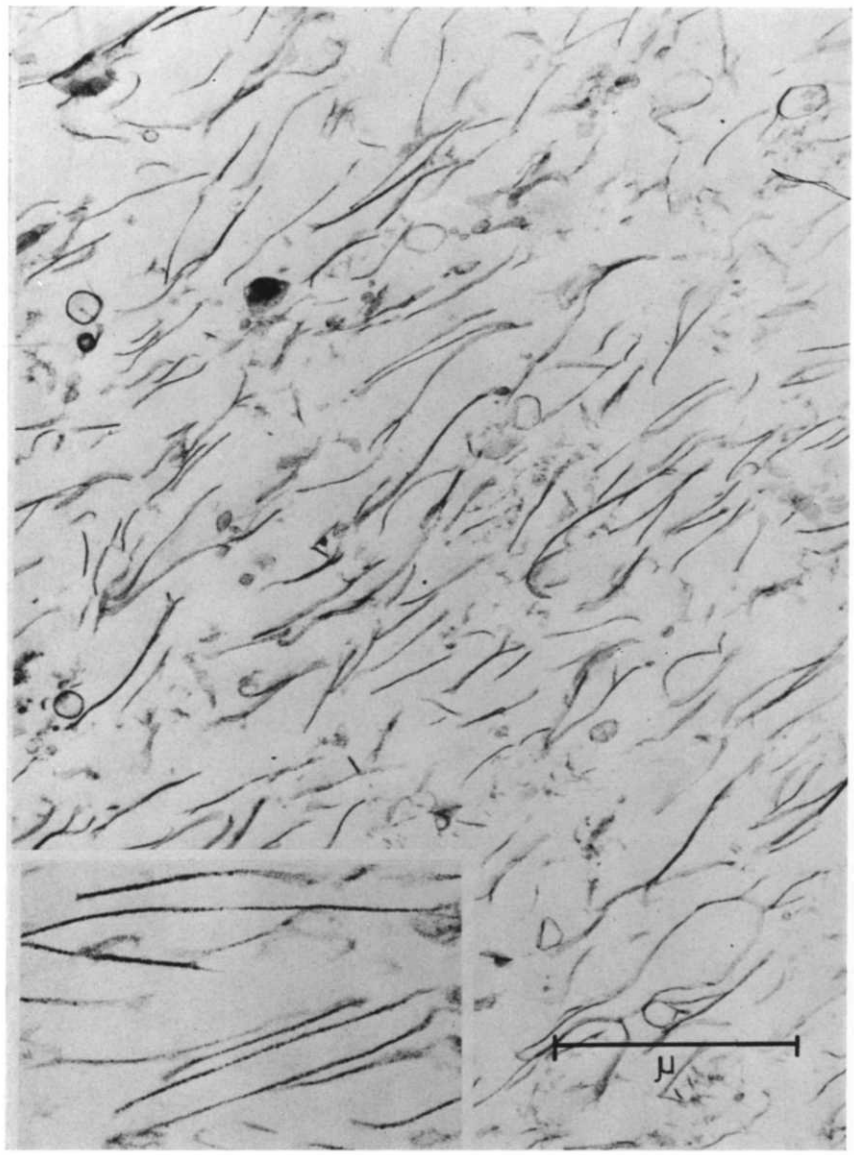

Fig. 5. Electronmicrograph of the phospholipase A_2-treated synaptic membranes. The picture shows Fraction 13. The inset is a high magnification photo of some of the membranes (× 300 000).

fraction II is between 2 and 3 %, whereas fraction 13 represents 0.04 % of the starting amount of protein.

DISCUSSION

The results show that phospholipase A_2 may be used for the preparation of membrane fragments, which retain acetylcholine receptor and 3′,5′-cyclic nucleotide

TABLE II

THE RELATIVE SPECIFIC ACTIVITIES OF THE MARKER PROTEINS IN FRACTIONS 11, 12 AND 13

Marker protein	Specific activity*		
	Fraction 11	Fraction 12	Fraction 13
Muscarinic receptor	37.9	4.6	17.7
Nicotinic receptor	0.2	3.8	15.8
3′,5′-cyclic nucleotide phosphodiesterase	27.1	0.15	16.7
Acetylcholinesterase	0.62	0.48	5.0
$(Na^+ + K^+)$-ATPase	2.1	1.3	9.25
Lactate dehydrogenase	0.62	0.83	0.92
Succinate dehydrogenase	2.1	0.41	0.28
NADH-cytochrome *c* reductase, rotenone insensitive	0.59	0.21	0.10
NADH-cytochrome *c* reductase, rotenone sensitive	2.3	0.71	0.25

* The specific activity found in the synaptic membrane fraction II was taken as unity. The absolute values of these units can be found in Table I. The data represent the means of 3–4 experiments, except for the nicotinic receptor and for the rotenone-insensitive NADH-cytochrome *c* reductase. In these cases the data are derived from duplicate experiments.

phosphodiesterase activity if the detergent effects of the formed lysophosphatidylcholine are abolished.

The membrane fragments obtained are rigid bar-like structures which cannot form vesicles. This was observed by Heilbronn and Widlund (unpublished) when phospholipase A_2 was originally used and is confirmed in the work by Cotman et al. [11]. The density of the present preparation corresponds to that of about 1.24–1.27 M sucrose, whereas Cotman et al. [11] reported that "postsynaptic densities" band at the 1.4-2.2 M sucrose interface. The discrepancy could be explained by the fact that the present procedure removes less than 20 % of the total phospholipids as compared to the 97 % lost upon *N*-dodecyl sarcosinate treatment [11].

The marker analysis showed that fraction 13 represents an approximately 15-fold enrichment in acetylcholine receptors and 3′,5′-cyclic nucleotide phosphodiesterase as compared to the synaptosomal membrane fraction. It is interesting to note that the muscarinic acetylcholine receptor and acetylcholinesterase are solubilized to a much higher extent than the other marker proteins. The reason for this may be that these two markers are exomembrane proteins. The fact that a number of marker proteins co-purified indicates that they may belong to the same membrane area.

It is always difficult to determine the origin of a membrane fragment solely by marker protein studies [26]. In the present case, a presynaptic junctional marker would have been of great value. Unless the high affinity choline carrier turns out to be specific for the presynaptic junction area, such a marker is not available. Use of neurotransmitter receptors as markers for postsynaptic structures requires special caution, in the light of recent findings on a possible presynaptic localization of adrenergic receptors [27], which were previously thought to be localized solely

postsynaptically. However, neither the fluorescent staining studies on the nicotinic acetylcholine receptor [28] nor those by labeling the muscarinic acetylcholine receptor with quinuclidinyl benzylate [29] showed any presynaptic cholinergic receptor. Therefore copurification of both cholinergic receptors with 3′,5′-cyclic nucleotide phosphodiesterase, which according to histochemical studies [15] is postsynaptically located, suggests an enrichment in postsynaptic structures. This suggestion is further corroborated by the peculiar morphology of the membranes, which appear as rigid fragments of the same size as the synaptic junction. Lack of presynaptic markers, on the other hand, is an obstacle in evaluating the amount of presynaptic membranes present in the fraction.

The present fraction seems to retain the outside regions of the postsynaptic membrane, which face the synaptic cleft. Thereby this fraction is a complement, in studies on postsynapses, to the postsynaptic density fraction [11], which represents the specialized inside regions of the postsynaptic membrane.

Further experiments are required to evaluate whether these membranes are functionally intact and whether the phospholipase A_2 treatment leads to some changes in their composition, either via fusion of membranes or due to adsorption of solubilized proteins.

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